Segregation of Total Carotenoid in High Level Potato Germplasm and Its Relationship to Beta-Carotene Hydroxylase Polymorphism

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ABSTRACT

High carotenoid potato may have particular value for human health due to the antioxidant properties and the therapeutic value for eye health in patients at risk for macular degeneration. Carotenoid concentrations were determined among the progeny of a cross between two high carotenoid lines derived from diploid *Papa Amarilla* germplasm from South America. The total carotenoid content ranged from 82 to 2686 µg / 100 g fresh weight (FW). The higher values greatly exceeded the mid-parent value of the cross. An index for yellow tuber flesh color was determined for a subset of the progeny. A cleaved amplified polymorphic sequence (CAPS) assay was developed to distinguish the alleles of beta-carotene hydroxylase (*bch*) in the two high-carotenoid parents. A *bch* allele (denoted B) common to the high carotenoid parents co-segregated with yellow flesh in the progeny of a white-flesh x yellow-flesh cross, making *bch* an excellent candidate for the classical *Y* locus, required for yellow tuber flesh. The same allele was also present in all other yellow-fleshed potato clones tested. Genotype at *bch* explained a portion of the variation of total carotenoid (R^2 = 0.42). Clones homozygous for the B allele (BB) contained, on average, slightly more carotenoid than heterozygous Bb clones, which in turn had much more carotenoid than homozygous bb clones, suggesting a partially dominant gene model. Similarly, bb flesh was significantly less yellow than Bb and BB, the latter two being quite close. Total carotenoid varied considerably between progeny in the Bb and BB genotype categories, suggesting that variation at one or more additional loci have a significant effect on total carotenoid levels. Since the total carotenoid levels in many *Papa Amarilla* cultivars and progeny are much higher than those in white- and yellow-fleshed tetraploid cultivars, it may be possible to breed for high carotenoid values in tetraploid germplasm by introducing one or more genes derived from *Papa Amarilla* germplasm.

RESUMEN

Las papas con alto contenido de carotenoides podrían tener particular valor en la salud humana debido a las propiedades antioxidantes y al valor terapéutico para la salud ocular, en pacientes con riesgo de degeneración macular. La concentración de carotenoides fue determinada en la progenie de un cruzamiento entre dos lineas con carotenoides, derivadas de germoplasma del diploide Papa Amarilla de Sudamérica. El contenido total de carotenoides varió de 82 a 2686 microgramos por 100g de peso fresco. Los mayores valores totales excedieron al valor del progenitor medio de los cruzamientos. Para un subconjunto de la progenie, se determinó el índice del color amarillo de la pulpa. Se desarrolló una prueba de división de secuencia polimórfica amplificada (CAPS) para distinguir alelos de beta caroteno hidroxilasa (*bch*) en dos de los progenitores

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con alto contenido carotenoideo. Un alelo *bch* (designado B), común en progenitores de alto caroteno co-segregó a pulpa amarilla en la progenie de un cruzamiento de pulpa blanca x pulpa amarilla, haciendo *bch* un excelente candidato para el clásico locus *Y*, requerido para pulpa amarilla del tubérculo. El mismo alelo estuvo también presente en todos los otros clones de pulpa amarilla probados. La explicación de la variación de una porción del total de carotenoide (R^2 = 0.42) la dio el genotipo *bch*. Clones homocigotas para el alelo B (BB) contenían en promedio, ligeramente mas carotenoide que los clones heterocigotas Bb, los cuales a su vez tienen mucho mas carotenoide que los clones homocigotas bb, sugiriendo un modelo de gen parcialmente dominante. Similarmente, la pulpa de bb fue significativamente menos amarilla que Bb y BB, siendo las dos últimas muy parecidas. El total de carotenoide varió considerablemente entre progenies en las categorías de genotipo Bb y BB, sugiriendo que la variación en uno o más loci, tienen efecto significativo sobre los niveles totales de carotenoide. Desde que el total del nivel de carotenoide en muchos cultivares y progenies de Papa Amarilla son mucho mas altos que en los cultivares tetraploides de pulpa blanca y amarilla, sería posible hacer cruzamientos para mayores valores de carotenoide en el germoplasma tetraploide, introduciendo uno o mas genes del germoplasma derivado de Papa Amarilla.

INTRODUCTION

Potato (*Solanum* spp.) tubers vary widely with respect to the types and concentrations of carotenoids they contain. White-fleshed clones contain low levels of carotenoid, while yellow-fleshed varieties contain more. The yellow flesh trait is considered to be controlled by a single locus (*Y/y*) mapped to chromosome 3 (Bonierbale et al. 1988). Even so, yellow-fleshed varieties vary greatly in degree of yellow coloration and concentration of carotenoid (Brown et al. 1993, 2005; Lu et al. 2001). The carotenoids in tubers are primarily xanthophylls; tubers contain only a trace of beta-carotene. The presence and concentrations of xanthophylls vary in different potato genotypes, with lutein predominating. Zeaxanthin, violaxanthin, and others have been reported in various studies (Iwanzik et al. 1983; Brown et al. 1993; Lu et al. 2001; Nesterenko and Sink 2003). The highest levels of tuber carotenoid are found in potato varieties of the Andes of South America, which are collectively known as *Papa Amarilla* (yellow potato). *Papa Amarilla* is represented by diploid cultivated potatoes *Solanum tuberosum* L. in the Groups Phureja and Goniocalyx. A number of reports of carotenoid content have contributed to the knowledge base in the last 60 years. These are summarized in Table 1. The first reports indicated that white-fleshed potato contained less than 100 µg / 100 g fresh weight (FW), while yellow-fleshed tubers contained more. Intensely yellow flesh was accompanied by levels reaching 560 µg. However, it is only in the last 15 years that very high levels of carotenoid have been recognized. Brown et al. (1993) reported wide variation in segregating genotypes derived from *Papa Amarilla* germplasm, with some clones exceeding 2000 µg. Several other studies have reported such high levels (Lu et al. 2001; Brown et al. 2005). Haynes et al. (1994) found that yellow color was more intense in smaller tubers. In a report by Brown et al. (1993), the existence of a so-called orange allele, *O*, was postulated for the *Y/y* locus. The effect of *O* was to confer accumulation of a high level of zeaxanthin in the tuber flesh. Heritability of yellow intensity (YIE313) was determined by Haynes (2000) to be extremely high (h^2 = 0.99), which also agreed with a well-expressed and highly heritable and possibly simple genetic component. Transmission of high expression of carotenoid by crossing the *O* allele into non-*Papa Amarilla* germplasm was not successful (unpublished breeding results) in producing progeny with high levels, forcing the search for better explanations of genetic control. The identification of possible candidate genes for the potato *Y/y* locus, based on mapping carotenoid pathway genes in other Solanaceae provides an opportunity to explore candidate gene polymorphism in popu-

TABLE 1—*Citations of total carotenoid content in potato flesh expressed as µg / 100 g fresh weight (FW).*

Concentrations (µg / 100 g FW) of total carotenoids in flesh	Citation
14-187	Caldwell et al. 1945
60	Brunstetter and Wiseman 1947
199-560	Kasim 1967
102-219	Tevini et al. 1984, 1986
27-329	Iwanzik et al. 1983
Segregation > 2000 (*Papa amarilla*)	Brown et al. 1993
97-536	Hale 2003
1435-2200	Lu et al. 2001
48-879	Nesterenko and Sink 2003
40-795	Brown et al. 2005

lations showing a range of carotenoids accumulation (Thorup et al. 2000; Hirshberg 2001). The present report provides additional information that incrementally improves our understanding of the genetic control of carotenoid level.

MATERIALS AND METHODS

Genetic Materials

The majority of the potato genotypes analyzed here were from a progeny between a cross between two high carotenoid lines (*Yema de Huevo* and 91E22). *Yema de Huevo* was obtained from Dr. Nelson Estrada, INIA, Tibaitatá, Colombia. The clone 91E22 was selected from crosses between clones originally obtained from populations derived from Groups Phureja and Stenotomum provided by Dr. Frank Haynes, Department of Horticulture, North Carolina State University, Raleigh, NC (Brown et al. 1993). True seed derived from the cross was soaked for 24 h in 1500 mg L^{-1} gibberellic acid (Sigma, St. Louis). Seed was sown in soil (Sunshine Mix, No. 1, Sun Gro Horticulture Distributing, Bellevue, WA), seedlings were transplanted to 20.3-cm-diameter plastic pots and plants were grown to maturity in a greenhouse. Tubers derived from this were planted after breaking dormancy. These tubers were grown in the same manner as the seedlings described above; tubers were harvested and stored at 4 C in 85% relative humidity. One month after storage, tubers were removed from storage and carotenoids were extracted from them. Two replicates (derived from separate pots) of each genotype were processed for carteonoid extraction and three replicates (from separate pots) were evaluated for yellow intensity. A related family was constructed by crossing 91E22 (as a male) with 07248-02. The latter clone was kindly provided by H. De Jong (Agriculture and Agri-Food Canada), and has only *Solanum tuberosum* group *tuberosum* in its immediate ancestry.

Beta-carotene Hydroxylase Cloning and Development of CAPS Assay

Potato EST accessions BI179773 and BM111217 are similar in sequence to the tomato beta carotene hydroxylase gene located on chromosome 3. Primers BCH-F1 (5'CCAGTTCTGTTCTTCTCTCCG) and BCH-R1 (5'TCAAAAGGTCCTTCTCTTGGTCTA) are based on these ESTs and were used to amplify genomic DNA isolated from 91E22, with the following thermal profile: 94 C for 2 min, followed by 35 cycles of (94 C, 15 s; 55 C, 15 s; 72 C, 60 s). This and other amplifications below were carried out in a volume of 50 µL and contained 200 nM of each primer, 200 µM dNTPs, 10 mM Tris-Cl pH 8.3, 50 mM KCl, 2.5 mM $MgCl_2$, 0.05% Nonidet-P40, 50-200 ng of template DNA, and 2.5 units of *Taq* DNA polymerase. The resulting PCR products were ligated into vector pGEM-T following the manufacturer's (Promega) instructions. Seven clones were obtained and sequenced, revealing two alleles that could be differentiated by virtue of a polymorphic *Taq* I restriction site. To monitor the segregation of these alleles in progeny, PCR was performed with primers BCH-F2 (5'CATGACATAGTTTGAATTTTGAGTC) and BCH-R2 (5'CGTTTGGCGCTGCCGTAAGTT), with the following thermal profile: 94 C for 2 min, followed by 35 cycles of (94 C, 15 s; 55 C, 15 s; 72 C, 30 s). Amplification products were then digested with *Taq* I.

Genomic DNA of cultivars and breeding clones was also evaluated with the CAPS assay. The following yellow-fleshed cultivars were evaluated: Amandine, Austrian Crescent, Banana, Bintje, Carola, Cynthia, French Fingerling, German Butterball, Keuka Gold, Ozette, Peanut, Penta, Regina, Rose Finn Apple, Rote Eersteling, Sandy, Sieglinde, Sylvia, Yagana, Yellow Finn, and Yukon Gold. The following white-fleshed clones were evaluated: AC Candy Cane, Allegany, Bake King, Cara, Chieftain, Chippewa, Dakota Rose, Dark Red Norland, Genesee, Green Mountain, IdaRose, Katahdin, Kennebec, Lenape, Marcy, Monticello, NY99, NY115, NY118, NY120, NYL235-4 (Prince Hairy), Purple 5, Red Pearl, Redsen, Rideau, Sebago, Stirling, and Superior. Primers YellowF1 (5'TAATTACTCCATTCTTTTGCT) and BCH-F2 (5'ATTTACATGACATAGTTTGAATTTTG) were used for allele-specific PCR, using the following thermal profile: 94 C for 2 min, follow by 45 cycles of 94 C, 15 s; 50 C, 30 s; and 72 C, 120s.

Carotenoid Extraction and Quantitation

Total carotenoid was extracted and quantitated using the methods reported by Brown et al. (2005), which contained minor modifications of procedures presented in Breemen (2001). Briefly, carotenoid was extracted in a chloroform: methanol phase separation, retaining the chloroform phase accumulated in two separations, drying and re-dissolving in methanol. Concentration was determined by optical density spectrophotometrically at 450 nm wavelength using the extinction coefficient for zeaxanthin in methanol. Total carotenoid was expressed as µg / 100 g FW.

Yellow Index

The yellow index was measured using a Colorimeter (Minolta Chroma Meter Cr-200, Minolta Corporation, Ramsey, NJ). Tubers were sliced midway between the apical and stem end, patted dry on a paper towel, and immediately evaluated by placing the aperture of the colorimeter against the cut surface. A yellow index (YIE313) (American Society for Testing Materials 1991; Haynes et al. 1994) was derived from the three chromaticity values, Y, y, and z, using the algorithm YI E313 = 100 × [(1-(0.8467[Y/y(1-x-y)])/y]. Three replications per progeny were measured.

Statistical Analysis

The segregation of *bch* alleles was tested by chi-square analysis against the model of a 1:2:1 ratio of b/b:B/b:B/B. One-way analysis of variance, single degree of freedom orthogonal comparisons, and population standard deviation were performed according to Steel and Torrie (1980). Regression analysis of *bch* genotype vs carotenoid content was performed using the regression procedure in SAS (version 9.1.3, Cary, NC) program. Regression analysis was performed of total carotenoid regressed on the yellow index using Microsoft Excel Trendline Function testing linear and curvilinear options. The function with the highest coefficient of determination was selected.

RESULTS

Thorup et al. (2000) have previously suggested that the potato *Y* locus on chromosome 3 may correspond to *bch*. To test this hypothesis further, both alleles of *bch* were sequenced from diploid 91E22. One *bch* allele, which we designate B, contained a single *Taq* I restriction site and gave a diagnostic product 233 bp in length after amplification and subsequent digestion with *Taq* I (Figure 1). The other allele, which we designate b, contained two *Taq* I restriction sites and yielded a diagnostic fragment 147 bp in length with our CAPS assay (Figure 1). Allele B was found to absolutely co-segregate with yellow tuber flesh color in 155 progeny (82 yellow: 73 white) of a cross between 91E22 and a white-fleshed *S. tuberosum* diploid, 07248-02 (Figure 1). The carotenoid content of 10 randomly selected yellow-fleshed clones of this cross averaged 389 µg / 100 g FW (range 236-620), while 10 white-fleshed clones averaged 153 µg / 100 g FW (range 107-204).

When the same CAPS assay was used to examine an additional 21 yellow- and 28 white-fleshed varieties and breeding clones, the fragment diagnostic for allele B was detected in all of the yellow-fleshed clones tested (Figure 2A and data not shown). The same restriction fragment was clearly absent in many white fleshed cultivars, e.g., Allegany and Superior (Figure 2A). Interpretation was nevertheless more difficult in other white-fleshed cultivars, e.g., Green Mountain and Katahdin, as the latter displayed a faint doublet of DNA fragments that migrated similarly to the 233 bp fragment of allele B (Figure 2A). Sequencing portions of *bch* from a cultivar (Bake King) that exhibited the doublet indicated that the doublet is most likely an artifact that results from recombination between alleles during PCR. One Bake King allele was found to contain two *Taq* I sites, while another allele contained none. Recombination between these alleles would be expected to yield two novel products, each with a single *Taq* I site. Consistent with the recombination hypothesis, the doublet was only observed in potato clones that contained both no-cut and two-cut *bch* alleles (Figure 2A and data not shown).

To further test for the presence of a unique allele of *bch* associated with yellow flesh color, but without complications arising from recombination, a pair of primers specific to allele B was also developed. Amplification with these allele-specific primers yielded an identically sized 397 bp product from all 21 yellow-fleshed cultivars and breeding clones tested (Figure 2B and data not shown). The same primers amplified no product from any of the 28 white-fleshed clones tested (Figure 2B and data not shown). These results provide strong circumstantial evidence that the *Y* locus codes for beta-carotene hydroxylase, although formal proof of this relationship will require complementation. Alternatively, it is possible that *bch* allele B and the dominant allele at the *Y* locus are tightly linked.

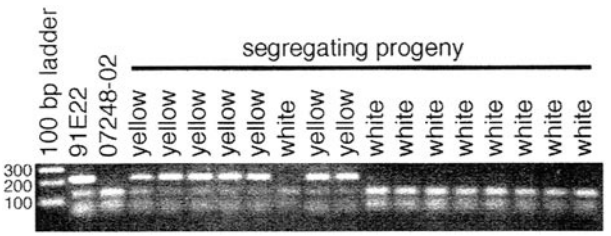

FIGURE 1.
Agarose gel showing that an allele of *bch* co-segregates with yellow tuber flesh in the progeny of cross between 91E22 (yellow flesh) and 07248-02 (white flesh). A fragment of *bch* was amplified by PCR and then digested with restriction enzyme *Taq* I. Products were separated on an agarose gel. A unique 233 bp fragment diagnostic of an allele we designate "B" can be seen in 91E22 and all progeny with yellow flesh.

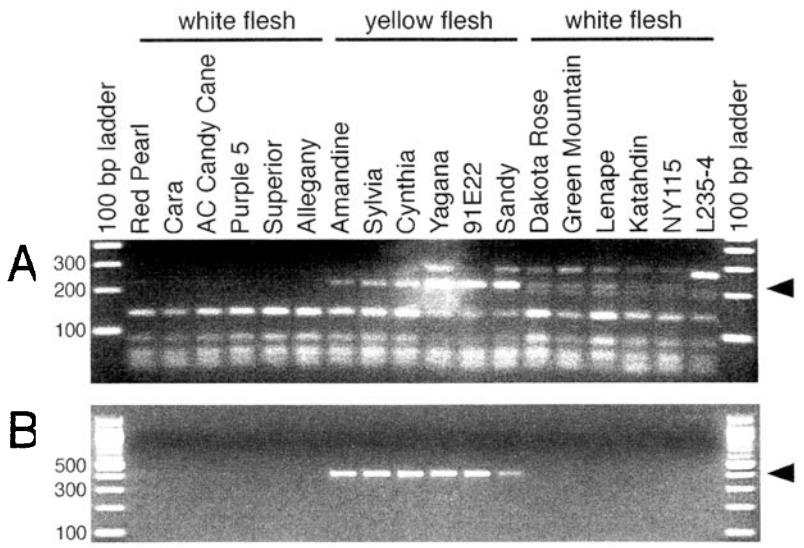

FIGURE 2.
Panel A. The CAPS assay illustrated in Figure 1 was applied to yellow- and white-fleshed varieties and breeding clones. The diagnostic allele B was detected in all yellow-fleshed clones. Allele B was clearly absent from the white-fleshed clones shown to the left, but it was difficult to determine if allele B was present in the white-fleshed clones shown to the right (partial results presented). Panel B. Primers designed to specifically amplify allele B were applied to the same clones as in panel A. These primers directed amplification of the expected 397 bp product only from yellow-fleshed clones (partial results shown).

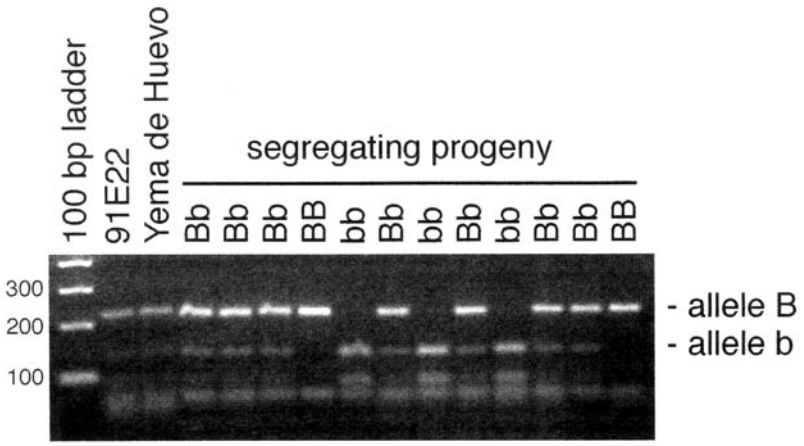

FIGURE 3.
The parents in the cross 91E22 × *Yema de Huevo* were heterozygous at the *bch* locus, each containing alleles B (one *Taq* I site) and b (two *Taq* I sites). The progeny conform to expectations of a 1:2:1 ratio of BB:Bb:bb from a Bb × Bb cross (partial results shown).

Exceptionally wide variation in tuber flesh color and carotenoid content was observed in the progeny of a cross between diploid parents 91E22 and *Yema de Huevo*. To further assess the relationship between *bch* and yellow flesh color, as well as between *bch* and carotenoid content, the co-dominant *bch* CAPS marker described above was used to evaluate the parents and progeny. As shown in Figure 3, both 91E22 and *Yema de Huevo* were heterozygous at the *bch* locus, each containing alleles B (one *Taq* I site) and b (two *Taq* I sites). The progeny of this cross are expected to segregate 1:2:1 for BB:Bb:bb. Actual numbers were 38:58:21 ($\chi^2 = 4.9$, $0.05 < P < 0.1$). Thus the genetic model of 1:2:1 segregation was not rejected. Total carotenoid values of the progeny span a large range, extending from 82 to 2686 µg / 100 g FW. This distribution, including the parental values (see arrows), are shown in Figure 4. The parents 91E22 and *Yema de Huevo* had carotenoid levels of 794 and 932, respectively. Some progeny far exceed the value of both parents, transgressive segregation is pronounced. The total carotenoid values of 12 progeny (13.5% of the population) were more than one standard deviation ($\sigma_{population}$ = 554 µg / 100 g FW) above the mid-parent (MP) value (MP = 863 µg / 100 g FW). The total carotenoids of three progeny exceeded the mid-parent value by two standard deviations.

The progeny are shown in Figure 5 grouped according to *bch* genotype in order of ascending total carotenoid value. There were significant differences between genotypes. Orthogonal comparisons planned *a priori* (Table 2) found significant differences in carotenoid content between all groups (i.e., b/b [$\bar{x}$ = 286] < B/b [$\bar{x}$ = 1059] < B/B [$\bar{x}$ = 1233]).

Tubers of 44 progeny, Yukon Gold (yellow flesh) and Russet Burbank (white flesh) were assessed for yellow index and carotenoid content. The yellow index (YIE313) showed a natural logarithmic relationship with carotenoid content (Figure 6). The colorimeter failed to measure greater yellowness above about 1000 µg. This

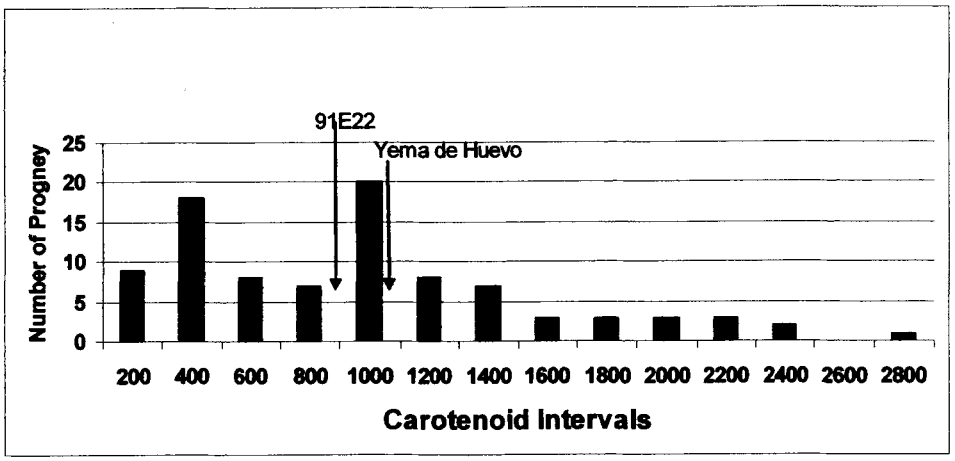

FIGURE 4.
Distribution of total carotenoid in 89 progeny of the cross 91E22 × *Yema de Huevo*. The range of the distribution suggests transgressive segregation.

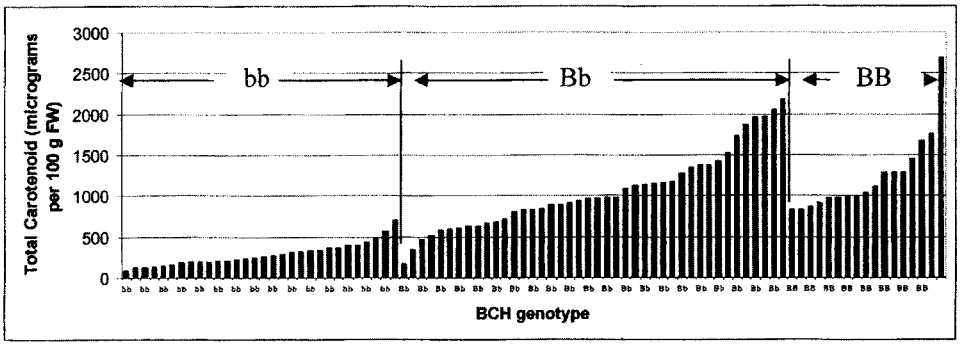

FIGURE 5.
Distribution of total carotenoids grouped by beta-carotene hydroxylase (*bch*) genotype. Variation in total carotenoids within the *bch* genotypes is very large. Orthogonal comparisons found significant differences between the groups bb, Bb, and BB.

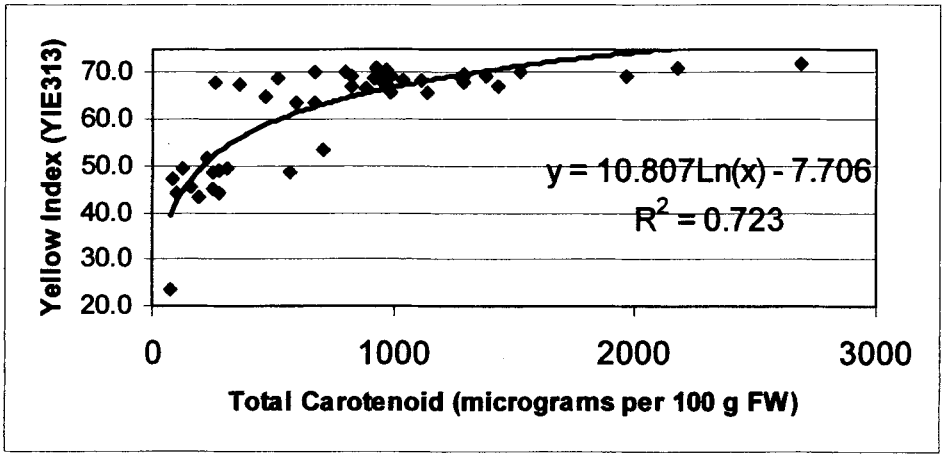

FIGURE 6.
Scatter plot of yellow index YIE313 in relation to total carotenoids. A natural logarithmic function more closely aligns with the data points than linear regression. The colorimeter failed to measure greater yellowness above approximately 1000 µg / 100 g FW.

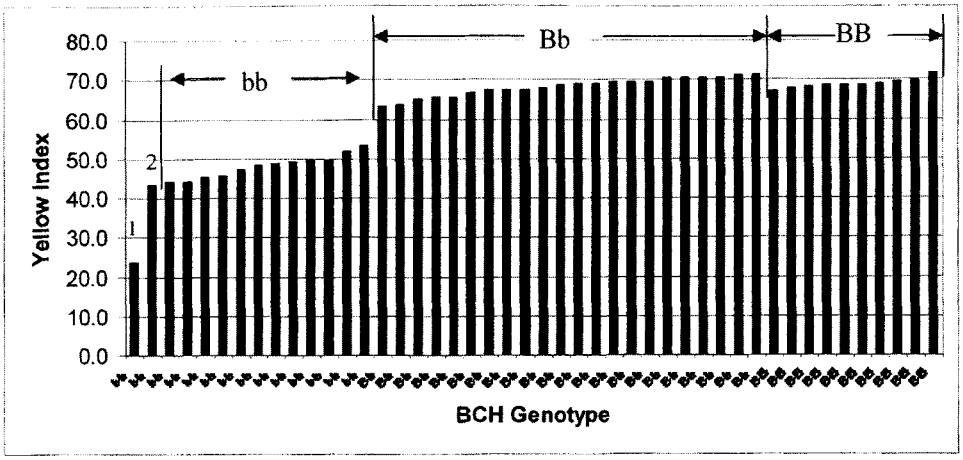

FIGURE 7.
Arrangement of yellow index (YIE313) values grouped according to beta-carotene hydroxylase genotype values. Orthogonal comparisons showed significant differences between all groups, although mean yellow indexes of Bb and BB are similar. Bars labeled 1 and 2 are Russet Burbank and Yukon Gold, respectively.

TABLE 2—*ANOVA and orthogonal comparsions of bb, Bb, and BB groupings of beta-carotene hydroxylase genotypes for total carotenoids (89 genotypes) and yellow index (44 genotypes). Progeny were derived from the cross of 91E22* × Yema de Huevo.

	Total Carotenoids		Yellow Index	
Source	df	Significance	df	Significance
Genotypes	88	$P < 0.001$	45	$P < 0.0001$
bb vs Bb	1	$P < 0.001$	1	$P < 0.0001$
Bb vs BB	1	$P < 0.001$	1	$P < 0.05$
bb vs BB	1	$P < 0.001$	1	$P < 0.001$
Error	89		92	

suggests that neither visual evaluation nor measurement by colorimetry will efficiently identify the highest levels (e.g., above 2000 µg) of carotenoid or differentiate them from the intermediate levels (1000 to 2000 µg). Extraction followed by optical quantitation will be necessary to select for the highest levels. The yellow index measurements of the progeny are presented grouped according to the *bch* genotype and ordered in ascending magnitude within group (Figure 7). Once again there were significant differences between genotypes. Three orthogonal comparisons found significant differences in the mean yellow index between the means of the bb, Bb and BB groupings (Table 2). The mean yellow index of bb (48.1) was significantly less than the means of Bb and BB genotype groupings (68.2 and 69.2, respectively).

DISCUSSION

Although *bch* polymorphism provides a loose explanation of the total carotenoid levels in the progeny of the cross studied here, the degree of variation within *bch* genotypes suggests that important contributions of other gene(s) will also be found for this population. Other polymorphisms, possibly within carotenoid biosynthetic genes like phytoene synthase and zeaxanthin epoxidase, need to be explored to obtain greater predictability and a more substantial association.

The highest levels of total carotenoid reported in the literature are all from germplasm derived from taxa in the Groups Phureja, Stenotomum and Goniocalyx that have been maintained in relatively closed populations at the diploid level without introduction of genes from other cultivated germplasm pools (Brown et al. 1993, 2005; Lu et al. 2001; Nesterenko and Sink 2003). These populations thus provide the most promising source for novel alleles that can be used to increase carotenoid content in cultivated tetraploid germplasm.

Römer et al. (2002) produced an increase of zeaxanthin in yellow-fleshed potato by transformation of sense and antisense constructs of zeaxanthin epoxidase. This inhibited conversion of zeaxanthin into violaxanthin. Increases in zeaxanthin over wild type ranged between four- and 130-fold. The highest levels of zeaxanthin reached 40 µg / g dry weight (approximately 1000 µg / 100 g FW). In a study of candidate gene expression of high (*Solanum phureja*) and low (*S. tuberosum*) carotenoids content, there was an inverse relationship between level of

zeaxanthin epoxidase transcript and carotenoids content during several stages of tuberization. Alternatively, relatively higher expression of RNA transcripts of certain enzymes in the carotenoid synthesis network in the early stages of tuberization (swelling stolons and developing tubers), was found for lycopene-ϵ-cyclase and isopentenyl pyrophosphate isomerase in *S. phureja* (*Papa Amarilla*) type compared to *S. tuberosum* cvs Pentland Javelin and Desiree (Morris et al. 2004). Identification of polymorphic alleles of genes encoding enzymes in the carotenoid pathway is the first step to elucidation of contribution to the high carotenoid phenotype characteristic in *Papa Amarilla*. The ability to transfer the high carotenoid trait from South American *Papa Amarilla* germplasm to cultivated North American and European tetraploid cultivars may require assembling a number of exotic alleles including the B allele of *bch* identified in this study.

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